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Origin and functional evolution of the Cdc48/p97/VCP AAA+ protein unfolding and remodeling machine

Dominik Barthelme[1,2] and **Robert T. Sauer**[1]

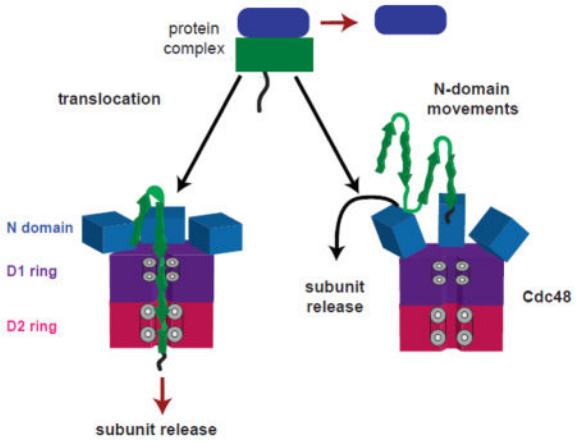
[1]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[2]Max-Planck-Institute for Infection Biology, Berlin 10117, Germany

Summary

The AAA+ Cdc48 ATPase (alias p97 or VCP) is a key player in multiple ubiquitin-dependent cell signaling, degradation, and quality-control pathways. Central to these broad biological functions is the ability of Cdc48 to interact with a large number of adaptor proteins and to remodel macromolecular proteins and their complexes. Different models have been proposed to explain how Cdc48 might couple ATP hydrolysis to forcible unfolding, dissociation, or remodeling of cellular clients. In this review, we provide an overview of possible mechanisms for substrate unfolding/remodeling by this conserved and essential AAA+ protein machine and their adaption and possible biological function throughout evolution.

Graphical abstract



1. Introduction

Members of the AAA+ protein family (ATPases associated with a variety of cellular activities) use common structural and operational principles to perform mechanochemical

work in cells, including protein unfolding prior to degradation, remodeling of macromolecular complexes, solubilization of aggregates, and translocation of proteins and nucleic acids.[1–4] Typically, AAA+ motor proteins assemble into ring shaped hexamers with an axial channel that can be used to translocate protein or nucleic-acid substrates. Here, we review recent progress in understanding the molecular mechanisms of substrate remodeling by Cdc48, a AAA+ enzyme that is also commonly known as p97 or VCP. In eukaryotic cells, Cdc48 plays roles in a bewildering array of cellular functions, including cell-cycle regulation, DNA replication, transport processes, immune signaling, reassembly of nuclear and Golgi membranes, and protein degradation by autophagy and by the ubiquitin-proteasome-system (UPS).[5–8] The ability of Cdc48 to remodel, unfold, or disassemble proteins and their complexes is thought to underlie each of these diverse activities.

2. Architecture and structure of Cdc48

Cdc48 is ubiquitous in eukarya and archaea and related enzymes are present in a limited number of eubacterial species. The functional form of Cdc48 is a homohexamer, assembled from subunits containing an N-terminal domain, two AAA+ modules (called D1 and D2), and a C-terminal tail (Fig. 1A). The body of the hexamer consists of stacked D1 and D2 AAA+ rings with a central axial channel (Fig. 1B–D).[9–11] The N-domain has two subdomains, a double Ψ-barrel and a four-stranded β-barrel. In eukaryotes, the N-domain has been implicated directly in substrate recognition, via binding of ubiquitin chains and possibly lipids attached to substrates,[12,13] and also acts as an interaction hub for numerous adaptor proteins (Table 1). These adaptors serve to recruit and/or modify additional protein substrates and regulate Cdc48 function by mechanisms that are still being determined.[14,15] Fig. 2 shows some of the pathways in which adaptors function. The tail of Cdc48 is largely unstructured and typically terminates with a hydrophobic residue (Hb), a tyrosine (Y), and a variable C-terminal residue (X). This HbYX motif mediates interactions with additional adaptor proteins (Table 1) and with the 20S proteasome (see below). Interestingly, the tyrosine in the HbYX peptide is phosphorylated in response to human T-cell receptor activation and other cellular stimuli, providing a mechanism to regulate binding of interaction partners to the C-terminal tails of Cdc48.[16–18]

3. Discovery and early functional studies of Cdc48

Cdc48 was first identified in 1982 in a genetic screen performed by David Botstein's lab for cold-sensitive yeast mutants defective in cell-cycle progression.[19] Subsequent purification and analyses of vertebrate Cdc48, which represents approximately 1% of all cytosolic proteins, revealed homology to NSF, a AAA+ ATPase required for vesicle fusion.[20,21] Indeed, Cdc48 was found to play an important role in homotypic fusion of ER membranes and to interact with clathrin molecules that mediate transport from the plasma membrane and Golgi complex to endosomal compartments.[22,23]

In seminal studies, Alex Varshavsky's lab discovered that proteasomal turnover of synthetic ubiquitin-fusion degradation (UFD) substrates required Cdc48 and the Ufd3 adaptor.[24] Cdc48 was subsequently found to be involved in multiple ubiquitin-mediated pathways, presumably as a consequence of binding ubiquitin chains directly or interacting with

adaptors that bind, ligate (E3 ligases), and/or remove ubiquitin chains (DUBs).[15,25–28] For example, Cdc48 plays a central role in the ERAD pathway (endoplasmic reticulum associated degradation).[29,30] To ensure quality control, an intricate surveillance machinery interrogates the folding status of proteins that transit the ER (~30% of all cellular proteins). Ultimately misfolded proteins are recognized by dedicated receptors and chaperone systems for retro-translocation into the cytosol for proteasomal degradation. In the case of luminal ER proteins, this process involves their transport through a protein-conducting channel thought to be formed by the ubiquitin-ligase Hrd1.[31,32] Depending on substrate topology and localization, different E3 ligases coordinate distinct ER degradation pathways, which ultimately require Cdc48 to forcibly pull the ubiquinated proteins out of the ER-membrane channel.[33] For this reaction, recruitment of cytosolic Cdc48 to the ER membrane depends on ubiquitin-binding cofactors (Ufd1 and Npl4) and an additional ER-tethering factor (Ubx2).[28,34,35] Studies of the fatty-acid regulation OLE pathway by Stefan Jentsch's lab introduced the concept of Cdc48 being an ubiquitin-selective segregase that remodels protein complexes or extracts client proteins from cellular structures such as membranes to allow proteolysis.[36,37] Although many facets of the diverse eukaryotic biology of Cdc48 have been studied and its activity has been shown to be essential in yeast and mice,[38,39] it is still largely unknown how this essential protein machine functions at a molecular level and how it forcibly remodels its cellular clients.

In contrast to the diverse biological functions established for eukaryotic Cdc48, relatively little is known about the physiological roles of Cdc48 in archaea, although biochemical studies of archaeal Cdc48 have established important enzymatic activities.[40,41] The presence of Cdc48 in archaea was first described in *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum*,[42,43] but it is now clear that archaeal Cdc48 orthologs are ubiquitous, often in multiple isoforms. For example, *Haloferax volcanii*, a genetically accessible model archaeon, harbors multiple Cdc48 genes, and attempts to delete *cdc48d,* one of four isoforms, failed, suggesting that Cdc48d is essential for cell growth and survival.[44] Archaeal and eukaryotic Cdc48 orthologs share high sequence identity (up to 60%) in the D1 and D2 AAA+ motor domains and less homology in the N-domain, although the overall fold of this domain is conserved between archaea and eukarya.[9,14] Bacterial homologs share even less overall sequence identity and are clearly more distantly related (Fig. 3). In both archaea and eukarya, Cdc48 is highly expressed during normal growth, is upregulated during stress, and is subject to posttranslational modifications.[45–50] Studies directed by Peter Zwickl and Wolfgang Baumeister established that purified archaeal Cdc48 could harness the energy of ATP-hydrolysis to unfold a model protein (GFP) and showed that both ATP hydrolysis and unfolding were markedly stimulated by deleting the N-domain, establishing that this domain can negatively regulate Cdc48 function.[40]

4. An archaeal Cdc48•20S proteasome

One class of AAA+ enzymes function in ATP-dependent protein degradation to maintain protein-quality control and regulate cell-signaling pathways. In these energy-dependent proteases, a ring-shaped AAA+ hexamer recognizes protein substrates, unfolds them, and then translocates the denatured polypeptide through an axial pore and into the chamber of an associated self-compartmentalized peptidase for degradation.[51,52] In one subgroup of AAA+

proteases, the self-compartmentalized peptidase is known as the 20S proteasome, based on its sedimentation coefficient, and consists of four stacked heptameric rings with an $\alpha_7\beta_7\beta_7\alpha_7$ topology.[53,54] The 20S enzyme functions with the Mpa AAA+ hexamer in actinobacteria, with the PAN AAA+ hexamer in archaea, and with the $Rpt_{1–6}$ AAA+ ring, which is part of the 19S regulatory particle of the 26S proteasome, to mediate degradation in the cytosol and nucleus of eukaryotes.[55] In each case, the outer α_7 rings mediate interactions with the hexameric AAA+ partner, whereas the inner β_7 rings harbor the proteolytic sites.

The PAN•20S proteasome was originally thought to be the only cytoplasmic AAA+ protease in archaea. However, the archaeal 20S peptidase is ubiquitous, whereas PAN is missing in ~15% of species and is dispensable for cell survival in some archaea in which 20S is essential, suggesting that an additional proteasome must exist.[41,56] Indeed archaeal Cdc48 was shown to interact with 20S and to function as an ATP-dependent protease.[53] As in other AAA+ proteases, the Cdc48 rings stack coaxially with the $\alpha_7\beta_7\beta_7\alpha_7$ rings, allowing proteins unfolded by Cdc48 to be translocated through its axial channel and into the 20S peptidase for degradation.[57] Deletion of the PAN gene in *H. volcanii* leads to an increase of the abundance of Cdc48 proteins, suggesting that these enzymes could have overlapping functions and substrate specificities.[49]

Two types of interactions stabilize assembly of the archaeal Cdc48•20S proteasome. In the first type, HbYX motifs in the C-terminal tails of Cdc48 dock into clefts on the α_7 rings of 20S. Similar HbYX-tail/α_7-cleft interactions also stabilize Mpa•20S, PAN•20S, and the 26S proteasome.[58,59] In each of these proteases, these HbYX docking interactions induce an open conformation of the axial gating portal that controls access to the 20S proteolytic chamber.[53,59–61] A second type of 20S-stabilizing interaction involves pore-2 loops at the bottom of the D2 AAA+ ring in archaeal Cdc48, which probably interact with axial loops in the α_7 ring but do not by themselves induce gate-opening.[62,63] Related pore-2 loop segments are present in Mpa, PAN, and the $Rpt_{1–6}$ ring and probably also function in mediating communication with 20S.[62,64] Interestingly, mammalian Cdc48 contains both the HbYX and pore-2 interaction motifs and can bind and open the gating portal of mammalian 20S.[62] However, binding is relatively weak (K_D > 1 μM), and a direct role for eukaryotic Cdc48•20S complex in proteolysis seems unlikely unless substrates or adaptors stabilize a Cdc48 conformation with higher 20S affinity.

5. Cdc48 and human disease

Mutations in human Cdc48 can lead to autosomal-dominant and ultimately fatal pathologies known as inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBMPFD) and amyotrophic lateral sclerosis (ALS).[65–68] Both diseases are characterized by the intracellular accumulation of ubiquitinated protein aggregates as a consequence of impairment of degradation by the 26S proteasome or autophagy. Most disease alleles are missense mutations that alter the interface between the N-domain and the D1 domain of Cdc48. These mutations can deregulate ATPase activity, affect the binding of selected cofactors, and change the structural orientation of the N-domain.[69–71] The N-domain normally packs in a co-planar manner against the periphery of D1 ring (Fig. 1), but different N-domain conformations are observed in crystal structures of disease mutants and

EM structures of wild-type Cdc48 with different nucleotides.[72–74] A minor group of Cdc48 disease mutations, which are also linked to familial ALS, affect the pore-2 loop of the D2 ring.[66,68] One such mutation ($Asp^{592}\rightarrow Asn$) alters binding of Cdc48 to human 20S but does not seem to affect other biochemical functions.[63]

6. Models for ATP hydrolysis and allosteric communication

How the two AAA+ rings of the Cdc48 engine couple conformational changes driven by ATP hydrolysis to the generation of force that allows unfolding/dissociation/remodeling of cellular clients is not known in molecular detail. Early studies suggested that the D1 ring does not hydrolyze ATP and serves principally to stabilize hexamer-assembly, whereas the D2 ring hydrolyzes ATP to support mechanical function.[75,76] It is now clear, however, that the D1 ring also hydrolyzes ATP, albeit at a substantially lower rate than the D2 ring.[77] The low ATPase activity of the D1 ring may result from very tight binding of the ADP product of hydrolysis. Crystal structures of full-length Cdc48 showed ADP bound to all six nucleotide-binding sites in the D1 ring, whereas nucleotide occupancy of the D2 ring varied with the crystallization conditions.[9,78] Solution studies confirm tight binding of ADP to the D1 ring and suggest that the D2 ring only binds 3–4 nucleotides tightly, as previously observed for the single AAA+ rings of other unfolding and remodeling machines.[79–81] The importance of ATP hydrolysis in both AAA+ domains of Cdc48 is underscored by genetic experiments that demonstrate that Glu→Gln mutations in the Walker-B motifs of either the D1 or D2 rings, which severely depress ATP hydrolysis, are lethal.[82,83] The D1 AAA+ ring may also play an important role in communicating allosteric signals from the N-domain to the D2 ring and *vice versa*. This signal transmission can be disrupted by mutations in the highly conserved sequences that link the N-domain to the D1 domain and the D1 domain to the D2 domain.[84,85] Indeed, cooperative interactions between these domains are essential for the physiological function of Cdc48.[86] Most studies addressing the ATPase activity of Cdc48 have been performed in the absence of adaptors or substrates, which could stimulate or repress activity and explain some results that otherwise seem contradictory.[87]

7. Models for substrate unfolding

Although ATP-fueled conformational changes in Cdc48 undoubtedly generate the mechanical forces that allow unfolding, dissociation, or remodeling of macromolecular complexes, the mechanism is still controversial. Studies from Tom Rapoport's lab on retro-translocation of the MHC class-I heavy chain suggested a model in which the protein is first engaged by the D1 ring and is then completely translocated through the axial channel and released into the cytosol for 26S proteasomal degradation.[82] In this instance, recognition appeared to involve interaction of the D1 ring with polypeptide segments of the substrate and interaction of the appended polyubiquitin chains with Ufd1 bound to the N-domain of Cdc48. This complete-threading model is similar to the well-documented mechanisms used by other AAA+ unfolding machines, including bacterial ClpX and ClpA and the proteasomal PAN and $Rpt_{1–6}$ motors, and is also supported by Cdc48 molecular-dynamics simulations.[88–90]

The threading model was challenged when the crystal structure of mammalian Cdc48 revealed a zinc-ion coordinated to multiple His^{317} side chains that severely constricted the axial pore of the D1 ring.[9] The authors of this paper reasoned that this constriction would block translocation and speculated that substrates might simply unfold by interaction with an external surface region of Cdc48 rather than by translocation through the axial channel. It is difficult, however, to reconcile a model of this type with extraction of a protein substrate from a membrane channel, which would appear to require repeated pulling events rather than a single remodeling event. An alternative model speculated that protein substrates might only be threaded through the axial pore of the D2 ring to avoid the D1-pore roadblock.[91] Consistently, mutations in or near the axial pore of the D2 ring of human Cdc48 were defective in ERAD degradation of TCRα (the α chain of the T-cell receptor), whereas mutations of residues in the axial pore of the D1 ring had little effect on degradation.[91] Trp551 and Phe552, two of the important D2-pore residues, form a canonical aromatic-hydrophobic motif characteristic of the pore-1 loops that grip substrates and transfer pulling forces, a hallmark of many well-studied AAA+ unfoldases.[92–96] In principle, substrates might enter the D2 pore without passing through the D1 pore by entering between the D1 and D2 rings or from the bottom of the D2 ring, although both of these possibilities seem unlikely.[91] Moreover, the residue that constricts the axial pore of the human Cdc48 D1 ring (His^{317}) is not conserved in archaea, fungi, or simple metazoans, and the D1-pore constrictions observed in crystal structures may not persist in solution during the mechanochemical cycle. Indeed, recent studies with a mutant variant of mammalian Cdc48 support substrate translocation through the full axial channel.[62,97]

Translocation-independent models for substrate unfolding and remodeling typically propose that pronounced movements of the N-domain and/or D1-D2 ring rotations, fueled by ATP hydrolysis, are responsible for local unfolding events on the Cd48 surface.[73,98–100] In such models, different parts of a protein substrate would bind to multiple regions of Cdc48 in one state, and nucleotide-dependent changes in enzyme conformation would then apply a distorting force to the bound substrate. Similar models have also been advanced to explain how SNARE complexes are disassembled by NSF[101–103], a AAA+ machine closely related to Cdc48. Such translocation-independent models may explain some Cdc48 remodeling events, but do not explain why the axial pore of the D2 ring should be important for Cdc48 some functions.[91] Moreover, archaeal Cdc48 clearly uses a translocation-based mechanism to unfold proteins and feed them into the degradation chamber of the 20S proteasome for proteolysis,[41,62,63] and single-particle EM reconstruction of the archaeal Cdc48•20S complex shows a continuous axial channel through which substrates could be translocated.[57] Interestingly, archaeal Cdc48 harbors canonical aromatic-hydrophobic pore-1 loops in the D1 and D2 AAA+ rings, whereas eukaryotic Cdc48 has this motif only in the D2 ring and has a Leu-Ala sequence at the corresponding D1-ring position. However, mutating this Leu-Ala sequence to the archaeal Tyr-Tyr sequence allowed the mammalian Cdc48 variant to unfold and translocate substrates through the axial channel and into 20S for degradation, suggesting that the machinery for translocation-based substrate unfolding remains intact.[62,97] In addition, a translocation-based unfolding mechanism has recently been proposed for a double-ring AAA+ enzyme that also contains a non-canonical pore-1 loop in the D1 domain.[104,105]

A hybrid model might explain the importance of pore loops for remodeling without requiring complete translocation of a substrate though the axial channel. Specifically, Cdc48 could use its pore loops to pull on a peptide segment of some protein substrates, resulting in substrate deformation and dissociation of a complex but not in unfolding or complete translocation. Indeed, ClpX, the AAA+ component of the ClpXP protease, normally unfolds target proteins and translocates them through its axial pore and into ClpP for degradation, but ClpX alone can also catalyze cofactor incorporation into metabolic enzymes in a reaction that requires ATP-hydrolysis and the axial pore loops but does not result in global substrate denaturation.[106,107] In the latter case, pulling presumably results in distortion but not global unfolding because of the mechanical stability of the target-protein structure. Hence, it is likely that mechanism by which Cdc48 remodels client proteins depends on the substrate and can also be regulated by adaptors and/or post-translational modifications.

8. Concluding remarks

General roles for Cdc48 in numerous aspects of eukaryotic cell biology have been well established. It is less clear, however, exactly how Cdc48 unfolds, dismantles, or remodels different client proteins to facilitate these biological activities. In addition the substrate selectivity of Cdc48 is very poorly understood. Do peptide sequences in the substrate need to get engaged by the axial channel of the Cdc48 ring? If so, what sequences are preferred? Reconstitution of Cdc48 activities in purified systems should allow biochemical and biophysical studies that address these questions and the detailed molecular mechanisms of remodeling.

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Highlights

- Eukaryotic Cdc48 plays essential roles in many ubiquitin-dependent cellular processes
- A large variety of adaptor proteins regulate Cdc48's diverse biological functions
- Archaeal Cdc48 functions with the 20S proteasome in ATP-dependent protein degradation
- Mechanistic models for client protein unfolding and remodeling by Cdc48 are discussed

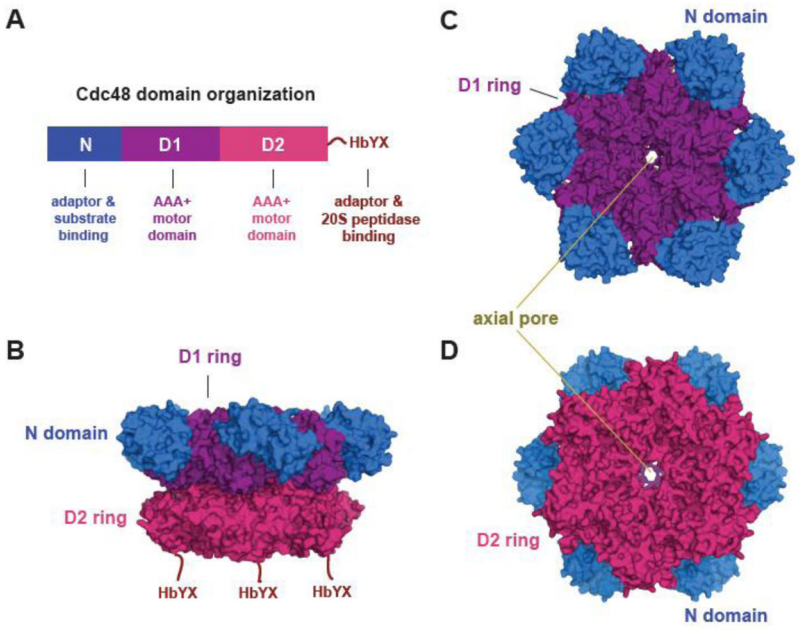

Figure 1.

Domain and three-dimensional structure of Cdc48. (A) Cartoon depiction of the domains of Cdc48. (B) Side view of the Cdc48 hexamer (pdb code 3CF1) shown in surface representation. (C) Top view of the Cdc48 hexamer shown in surface representation. (D) Bottom view of the Cdc48 hexamer shown in surface representation.

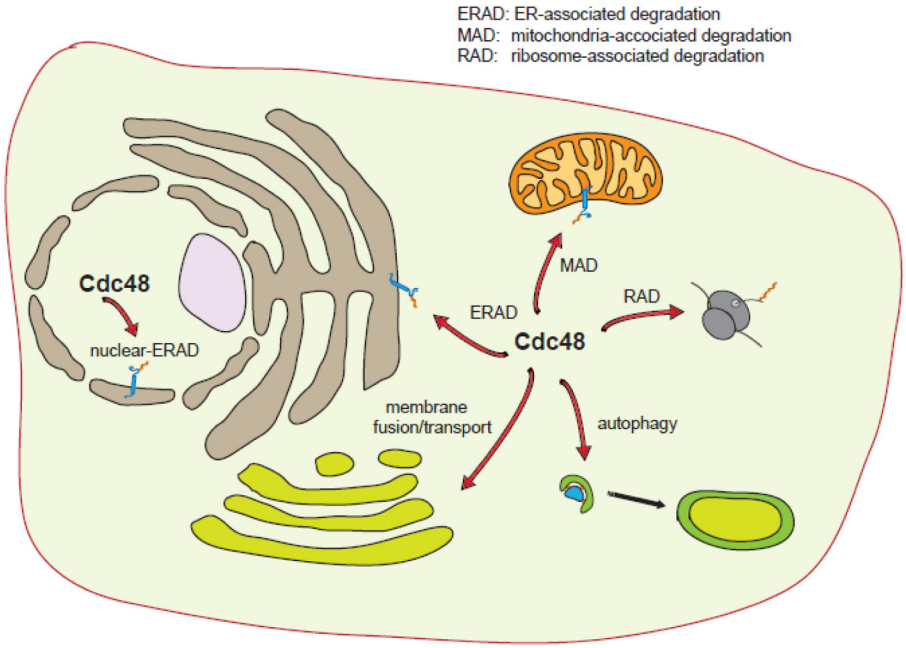

Figure 2.

Schematic view of a eukaryotic cell and numerous pathways in which Cdc48 and its adaptors have been implicated.

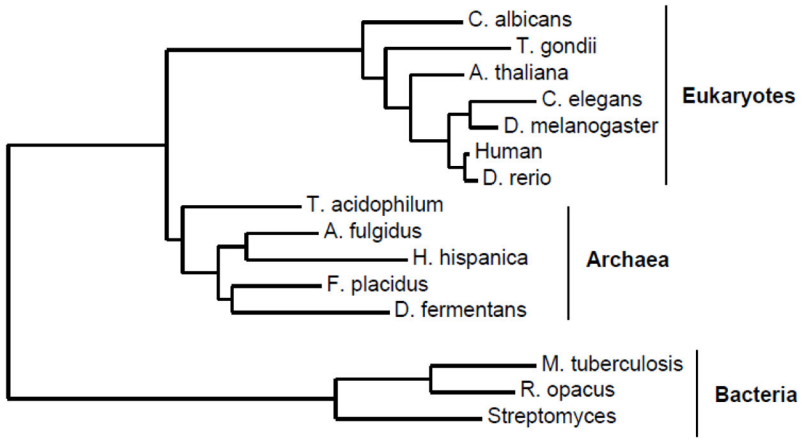

Figure 3.

As shown in this phylogenetic tree (created at http://www.phylogeny.fr), Cdc48 enzymes are found in all three domains of life. The bacterial enzymes are most distantly related.

Table 1

Selected proteins that function as adaptors for eukaryotic Cdc48.

adaptor	binding motif	Cdc48 binding region	associated function
Ufd1-Npl4	BS1/UBX-L	N-domain	ERAD; mitochondria-associated degradation; ribosome associated degradation; cell-cycle regulation; DNA repair
VMS1/ANKZF1	VIM	N-domain	mitochondria-associated degradation; ERAD
Shp1/p47	UBX/BS1	N-domain	autophagy; reassembly of nuclear and golgi membranes
Otu1/YOD1	UBX-L	N-domain	deubiquitinating enzyme; ERAD
Ataxin-3	VBM	N-domain	deubiquitinating enzyme; linked to Machado-Joseph disease; degradation of misfolded chaperone substrates
Ubx2/UBXD8	UBX	N-domain	lipid droplet homeostasis; ERAD; RNP complex remodeling
Ufd3/PLAA	PUL	HbYX tail	ubiquitin homeostasis; degradation of UFD substrates
PNGase	PUB	HbYX tail	removal of N-linked oligosaccharides from glycoproteins; ERAD
RNF31	PUB	HbYX tail	E3 ubiquitin ligase; NF-κB signaling
UBXD1	PUB VIM	N-domain HbYX tail	ERAD; endosomal trafficking and autophagy